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(54) Title: A NOVEL FAMILY OF PROTEASE INHIBITORS, AND OTHER BIOLOGIC ACTIVE SUBSTANCES			
(57) Abstract <p>The invention relates to novel protease-inhibitors which are obtainable from leeches. It also relates to uses thereof, for instance as a medicament, thus pharmaceutical preparations are provided, as are derivatives, mutants, genes encoding, vectors comprising and cells provided with such genes and/or vectors. In particular the invention relates to a family of proteinaceous protease inhibitors having a molecular weight of about 5.5kD and the primary sequences mentioned below. This invention also relates to HIV-inhibitors and other therapeutically interesting, low molecular weight, and low antigenic substances from leeches.</p>			
DDNCGGKVC SKGQLCHDGHCECTPIRCLIFCPNGFAVDENGCELPCSKKHQ DDDCGGQVC SKGQLCVDGQCKCTPIRCRIYCPKGFEVDENGCELPCTCLQ DGNCGGQVC SKGQLCVDGQCKCTPIRCRIYCPKGFEVDENGCELPCTCLQ			

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Title: A novel family of protease inhibitors, and other
biologic active substances

The present invention relates to certain novel compounds which have protease inhibitory activity, as well as compounds derived therefrom and compositions comprising such compounds, and other biologically active substances. More specifically the invention relates to such compounds which are polypeptide-like
5 or of a proteinaceous nature and of polysaccharide derivatives and/or glyco-poly saccharide. The invention especially relates to such compounds and compositions and uses thereof obtainable from leeches.

10 The invention further relates to therapeutic uses of the novel protease inhibitors. An illustrative example of such a use is given below.

Several diseases, like emphysema, arthritis, gingivitis, periodontitis and other inflammatory conditions are associated
15 with tissue destruction caused by the enzyme human neutrophil elastase (HNE). HNE is a serine protease which is capable of solubilising fibrous proteins like elastin and collagen. HNE is mainly present in the azurophilic granules of neutrophil leucocytes. Under normal physiological conditions, the
20 proteolytic activity of the enzyme is kept under control by an excess of inhibitors present in plasma and other secretions. However, some disorders result in a local deficiency or inactivation of inhibitor which leads to an imbalance in the ratio of inhibitor to elastase, resulting in tissue
25 destruction.

The balance may be restored employing protease inhibitors, for instance those provided by the invention.

Furthermore, in the replication cycle of HIV, the proteolytic cleavage of the gag and env precursors is an
30 important step. Development of inhibitors of these proteases is a rationale in anti-viral drug development. Study-objectives evaluating anti-viral capacity of potential substances is therefore valuable.

Various substances extracted from leeches are known to
35 have useful biological activity. These were reviewed by Sawyer,

(Sawyer, 1990). Essentially ~~two groups~~ of activity can be recognised. The first group comprises antithrombotic and fibrinolytic activities, the second group comprises enzymes and inhibitors. Well known representatives of the first group
5 include for instance Hirudin, a thrombin inhibitor, (Markwardt, 1956; 1988; Petersen, et al, 1984); Hementin, a fibrinolytic agent (Budzynski, et al, 1981; Kirschbaum & Budzynski, 1990); Antistasin, an inhibitor of coagulation factor Xa (Gasic, et al, 1983), which was reported to have antimetastatic properties
10 as well; Gilanten, another factor X inhibitor (Condra, et al, 1989; Blankenship, et al, 1990). Representatives of the second group are: Bdelein, an inhibitor of trypsin and plasmin (Fritz & Krejci, 1976); Eglin, an inhibitor of chymotrypsin, elastase and Cathepsin G (Seemuller, 1979); Orgelase, an hyaluronidase
15 (Sawyer, 1986).

More recently several additions in this field have been published in patent literature: A fibrinolytic enzyme isolated from *Hirudo medicinalis*, which splits Glutamyl-Lysin sequences (EP 0502876); a platelet adhesion inhibitor, isolated from
20 *Hirudo medicinalis*, which inhibits collagen-induced platelet aggregation (EP 0552269); a thrombin inhibitor from the leech *Hirudinaria manillensis* (PCT/GB89/01345); an inhibitor of platelet aggregation from leeches from the *Hirudinidae* family (EP 0348208); an anticoagulant/modulator factor isolated from
25 *Hirudo medicinalis* (EP 0352903); A chymotrypsin- and elastase inhibitor from *Hirudinaria manillensis* (PCT/NL90/00046).

This invention provides novel protease-inhibitors and other biologically active substances, as well as pharmaceutical and cosmetic preparations containing one or more of these
30 compounds. In one aspect the invention provides substances having protease-inhibiting activity obtainable from *Limnatis Nilotica* or fragments or derivatives of such substance having similar activity.

Such substances can be derived from all body parts and
35 secretions of the leech, inclusive saliva and gut-, intestinal- and skin secretions and mucus.

These novel Elastase/chymotrypsin- and trypsin-inhibitors according to part of this invention can be typically isolated from leech tissue by solvent extraction-techniques; alternatively they may be isolated from leech secretions (such as leech saliva), although the invention is not limited to specific ways of obtaining the novel protease-inhibitors. In a further embodiment the invention provides such a novel protease inhibitor which is of a proteinaceous or polypeptide-like nature. Preferably said proteinaceous substance includes at least a part of the following amino acid sequence: (1) (N-terminal) Asp-Asp-Asn-Cys-Gly-Gly-Lys-Val-Cys-Ser-Lys-Gly-Gln-Leu-Cys-His-Asp-Gly-His-Cys-Glu-Cys-Thr-Pro-Ile-Arg-Cys-Leu-Ile-Phe-Cys-Pro-Asn-Gly-Phe-Ala-Val-Asp-Glu-Asn-Gly-Cys-Glu-Leu-Pro-Cys-Ser-Cys-Lys-His-Gln : (Carboxy-terminal) or (2) Asp-~~Asp-Asp~~-Cys-Gly-Gly-Gln-Val-Cys-Ser-Lys-Gly-Gln-Leu-Cys-Gly Asn Val-Asp-Gly-Gln-Cys-Lys-Cys-Thr-Pro-Ile-Arg-Cys-~~Arg~~-Ile-Tyr-Cys-Pro-Lys-Gly-Phe-Glu-Val-Asp?-Glu-Asn-Gly-Cys-Glu-Leu-Pro-Cys-~~Thr~~-Cys-Lys?-Gln? although it will be clear that the activities are the really important features of this invention, so that mutations, isoforms, derivatives, such as salts, fragments or even peptidomimetics and anti-idiotypic or catalytic antibodies are also a part of this invention.

For the definition of isoforms or mutants, one has to understand that by biologic evolution enzymatic and other systems active molecules are subject to continuous phylogenetic development. It is in this understanding, that we define isoforms or mutant forms of these molecules. The primary structure (1) as reported here is one of three or four

isoforms, which have large conformity, and are being defined by molecule weight and amino acid sequence. The same may be true for (2).

A number of isoforms of (1) are identified below. The novel
5 protease-inhibitors can be applied in the known applications for such substances. They can be suitably formulated into pharmaceutical compositions, which may comprise suitable excipients for administration. Administration may be accomplished in any suitable manner, although for proteinaceous
10 substances in systemic applications parenteral routes are preferred. Dosages for these substances can be taken from the literature and designed on the basis of specific activities of the substances, the molecular weight of these substances, the weight of the subject to be treated, the kind of application, etc. Dosages will usually lie between 0.1 µg/kg and 10 mg/kg
15 bodyweight.

Nucleic acid molecules encoding substances according to this invention are also provided. They can be used for detection of the gene encoding the substance, for expression of
20 the substance in suitable host cells and for preparing site-directed mutants. Site-directed mutations are often useful in that they can increase activity and/or stability of the encoded substance.

There are many suitable expression systems for expression
25 of substances according to the invention. Although expression in host cells is preferred, it is also possible to employ cell-free expression systems. Suitable host cells may be prokaryotic or eukaryotic since it appears that the proteinaceous substances according to the invention are not glycosylated or
30 modified post-translationally in any other way, although a signal sequence may be present. Usually a nucleic acid to be expressed is provided in a vehicle for expression, such as a vector, whereby regulatory elements are provided, such as promoters, enhancers, and the like.

35 A nucleic acid molecule and some alternatives thereof which encode a substance according to the invention are given below:

CTR CTR TTR ACR CCN TTY CAN ACR AGN TCR TTY CCN GTY AAY GAN
 ACR GTR CTR CCN GTT ACR CTY ACR TGN GGN TAD GCN TCY ACR AAY GAN
 TAD AAR ACR CGN TTT CCN AAR CGN CAN CTR CTY TTR CCN ACR CTY AAY
 GAN GGN ACR AGN TGT ACR TTY GTR GTY

- 5 whereby R=A/G, N=C/G/T, Y=C/T, D=A/G/T,
 or a sequence having 70% homology therewith.

Experimental

- 10 During applicants' routine research work with cultures of
 various bacteria-species, screening methods for new antibiotics
 were used. While researching on the bacterium *Aeromonas*
hydrophila species which is a common bacteria from freshwater
 fish, waterfowl and leeches, an "auto"-antibiotic effect on
 15 *Aeromonas* isolated from leeches was surprisingly found. Blood
 nutrient plates seeded with monocultures of *A. hydrophila* showed
 blank and developing areas, where seeding was performed. It
 appeared that the blank areas were contaminated with some
 substances from the host (leeches), which prohibited bacterial
 20 growth in the contaminated areas. After further searching this
 activity seemed to arise from body-derived substances of the
 leech under investigation, which was harbouring the colony of
Aeromonas. Applicant subsequently identified the original
 secretive elements of the leech body, isolated and purified the
 25 said factors, and discovered other biologic activities from
 this family of substances, which was named Fahsin. It was soon
 clear, that besides the said antibiotic effect, the substance
 was also biologically active as a protease inhibitor. The
 species of leech in which the substance was found belongs to
 30 the family of hirudinidae, and was designated as *Limnatis*
nilotica. The said purification of the substance finally
 revealed a new family of proteins, which was not earlier
 described. This family of proteins comprises 50/51 amino acids,
 and they occur in various isoforms, where substitutions have
 35 taken place in the structure at various places as described.

Other substances having a low antigenic or non-antigenic
 effect when used as therapeuticum were isolated.

L Nilotica (Savigny, 1820, as following from Autrum 1936) is described as a "nasal leech" or horse leech (Mouquin-Tandon 1846). It was signalled to be present in the whole littoral area of the Mediteranean (Harant, 1929; Jarry, 1959). It
5 lives in spring fountains and "oueds", and feeds on cattle, dogs and man (Blaise, 1874/5; Neveu & Lemaire, 1938; Turner, 1969; Keegan, et al, 1970).

Amazingly, the feeding habits of this leech differ from other haemotophagous leeches. It remains attached to its host
10 (nasal - and laryngeal cavity) for prolonged periods (weeks subs. months). The animal feeds whenever it feels like doing so on its host repeatedly. We have observed that drinking cattle was infected with these leeches, which did not drop off while the cattle was drinking water. Only thick, fat, adult-
15 size leeches do drop off at such occasions. Therefore, it is clear that this species of leech is mostly free of antigenic or immunogenic substances in its mucus or salivary gland product. Reports of host animals dying from this species of leech mention anaemia as a common cause, but to our knowledge
20 no direct antigenic effect has been described.

The cycle of development of these leeches from hatchling to the reproductive phase spans over a few months in summertime only (Ghedia, 1984).

It was further observed, that these leeches, held in
25 captivity, could be fed by heparinized blood from the slaughterhouse in animal gut preparations. Sacrificed leeches which did not feed on blood for several months, still contained more or less liquid blood in crop and gut. Therefore, these leeches must produce anticoagulant
30 substances.

We further observed that this species of leech, held in captivity, was able to feed on blood clots. Several leeches did grow from hatchling to full-size leech under this regimen. After feeding, the remains of the blood clots are literally
35 pierced. Therefore, we believe, that these leeches also produce substances to dissolve blood clots, f.e. by dissolving fibrin.

The present description reveals the unique primary sequence of the protein of formula (1), subject of this invention, its isolation and purification, its specific activities, its production through gene cloning and expression. Applicant specifically intends that this protein Fahsin, as invented, and described herein, includes such substance however produced, be it through sequential and on block synthesis or through gene cloning and expression.

The present invention also provides the primary sequence of the proteins/peptides of formula (2) and its specific activities (especially the inhibition of trypsin and plasmin). These peptides belonging to the Fahsin family of course also belong to the invention, regardless of their origin or way of production.

Description of isolation .

1. Frozen *Limnatis nilotica* (300 g) were dehydrated in 94% ethylalcohol at room temperature: using three changes of total 400 ml. 100 ml of the ethanol extract was lyophilized in vials, after adding 300 ml destillated water. (One can also, as an alternative, use the chopped heads of these leeches, or use activated mucus secretions from the live leeches, by immersing them for one hour in 150 mM NaCl, 10 mM Arginine and 20 mM Phosphate buffer, at pH 7.0 at room temperature, or immersing the live leeches in 4% ethylalcohol for ten minutes, and collecting the large mucus secretions then produced) and thereafter lyophilized). All methods resulting in obtaining lyophilized base material.
2. Prior lyophilized base material underwent solubility tests after resuspension of the base material in 1) 0.1 M Acetic Acid, 2) 50% Acetic Acid and 3) 0.1 M Ammoniumbicarbonate and after centrifugation of the various suspensions, supernatants and pellets (after resuspension). Thereafter these were tested on the presence of protein by analytical sequence analysis (Edman degradation with automated sequenator Applied Biosystems, Model 477A) (Edman, 1956; Ilse

& Edman, 1963), by applying protocols, reagentia, chemicals and materials from Applied Biosystems (Warrington, UK and Foster City, Cal, USA) (Hewick, et al, 1981). It was investigated if biologic activity included anti-elastase as the described "auto-antibiotic effect" of *A. hydrophila*. Therefore, tests on inhibition of elastase activity were executed with dried samples of 20 μ l of supernatant and resuspension of pellet of the base material on elastase (Boehringer) and SAAAP (Fluka) as chromogenic substrate, and 0.1 M Tris/HCl pH 8.2 as assay buffer. Based on the results obtained with the different solutions, and the inhibition of elastase, it was decided to use 0.1 M Hac as solution for the lyophilized base material and thereafter as eluens for gel filtration.

3. 15% of the base material was resuspended in 0.1 M Hac, centrifuged twice and reassembled (volume ca. 28 ml). This was thereafter passed on a Sephadex G-75 column (length 180 cm, diameter 1.85 cm, eluens 0.1 M Hac, fraction size ca 5 ml, flow adjusted at ca. 0.75 ml/min. A total of 180 fractions were sampled and absorbtion measured at 233 nm and 280 nm (for results see Figs. 1 and 2).

Tests on inhibition of elastase activity were measured every 4 fractions on fractions no's 48 to 180, in accordance with the measurements under 2 hereabove. Inhibition was found at fractions no's 92 to 116. (Fig. 3).

An analytical test sequence analysis was run on 200 μ l from fraction no. 105 in order to estimate the quantity of protein available. Signals present indicated a level of ca. 50 pmol, which indicates proteins/peptides at a level of 2.5 nmol, accepting 50% from initial yield.

A further estimate of molecular mass was performed on fractions from the Sephadex column with SDS-Page. Results are presented on gels no 1, 2 and 3 (Figs. 4, 5 and 6). Fractions 50, 60, 70, 80, 90, 100 and 110 of the Sephadex G-75 gelfiltration were used for Gel 1 (Fig. 4), fractions 88, 91, 94, 97, 100 and 103 were used for Gel 2 (Fig. 5) and fractions 106, 109, 112, 115, 118 and 121 were used for Gel 3

(Fig. 6). It was found that in the area of elastase inhibition (from fractions 92 to 116) molecular masses were estimated at ca. 5 kDa (see fraction 106 and 109 in Gel 3).

A pool was thereafter made from fractions containing no's 5 95 to 113 for further studies with:

4. Anhydrochymotrypsin column chromatography was performed on the previously pooled fraction no's 95 to 113. This pool was suspended in 6 ml 0.05 M Tris/HCl buffer, pH 8.2, containing 0.02 Ca Cl₂ and 0.02 % Na-azide, thereafter 10 centrifuged. The remaining pellet was resuspended with 50 µl 8M ureum plus 950 µl H₂O. 10 µl of the resuspended pellet and 10 µl supernatant were passed for analytical sequence-analysis. After comparison of the results, it was concluded, that 5 - 10% of the protein as found in supernatant was 15 present in the pellet. Inhibition of elastase could only be demonstrated with supernatant. Therefore, further proceedings took place with supernatant only.

A portion of 50% of the pooled fractions 95 to 113 (ca. 3 ml) was chromatographed over 1.5 ml "immobilized 20 Anhydrochymotrypsin" (Pierce, Rockford Ill, USA, nr 20185) over a polystyreen-column (Pierce, Rockford Ill. USA), following the instructions from the manufacturer (binding buffer: 0.05 M Tris-HCl buffer, pH 8.2, with 0.02 M CaCl₂, 0.02% Na-azide; first elution buffer: 0.1 formic acid, pH 2.5; 25 second elution buffer: 0.05 M Tris-HCl, pH 8.2, with 0.02 M CaCl₂, 0.02 Na-azide and 5 M NaSCN; flow ca. 9.2 ml/hr; temp. 4 °C, fraction size: ca 2 ml). The portion was eluted, after loading, with 30 ml binding buffer (fractions 1 - 17), 30 ml first elution buffer (fractions 18 - 31) 15 ml second elution 30 buffer (fractions 32 - 40) and 6 ml binding buffer (fractions 41 - 43).

These fractions were than tested on elastase inhibition activity by the standard elastase assay (as follows)

Elastase inhibition assay

The principle of the assay resides in inhibition of elastase (Boehringer, 1027891) activity on the chromogenic substrate SAAAP (Fluka, nr. 85975) as measured at 405 nm spectrophotometrically by monitoring release of the p-nitro-aniline group during substrate digestion.

Preparation of Solution:

- 10 Assay buffer: 0.1 M Tris/HCl pH 8.2, 1 M NaCl.
Elastase: 40 µg/ml in H₂O.
Substrate: 1.0 mM SAAAP in H₂O.
Glacial Acetic Acid 5%: diluted in H₂O.

15 Assay procedure:

Reaction mixtures (Eppendorf), containing 100 µl assay buffer, 50 µl sample elastase inhibitor, 50 µl elastase solution was started by addition of SAAAP (25 µl, 1 mM) and
20 incubated at 25°C for 30 minutes. Thereafter the reaction was stopped by addition of 25 µl 50% Glacial Acetic Acid. The Absorbance of released p-nitroaniline was read at 405 nm.

5. Inhibition of elastase activity was found only in
25 fraction 19 of the tested portion. Volume of this fraction was ca. 1900 µl. For control of the purity and the quantity of protein analytical sequence analysis were performed, resulting in indications that a mixture of peptides is present at a level of 1887 pMol, under assumption of an initial yield of
30 50%.

A chromatogram analytical HPLC of fraction 19 is presented in Fig. 7. Thereafter samples of peaks 5, 7, 8 and 9 were tested on elastase inhibition activity as described in the elastase inhibition assay procedure; only fractions 7, 8 and 9
35 showed inhibition of elastase. Fraction 5 did not show anti-elastase activity but a strong trypsin inhibition (see below).

6. Mass Spectrometry was performed with Laser Desorption. Within an accuracy of 0.1% the following masses were found for these fractions:

Fraction 5: 5377.2 and 5435.5 D (1:1 mixture)

5 Fraction 7: 5494.4 D.

Fraction 8: 5476.5 D.

Fraction 9: 5385.5 and 5454.1 D (1:1 mixture).

We have named these substances as follows:

10

Fraction 5: FAHSIN T 1/2

Fraction 7: FAHSIN E 1

Fraction 8: FAHSIN E 2

Fraction 9: FAHSIN E 3/4.

15

7. Characterization with protein sequence analysis.

Peak 7, 8 and 9 were analysed with standard Edman degradation. Various digestive steps (trypsin digest, NaOH incubation for

20 Asn-Gly split, Glu-enzyme digest) led to the complete sequence of fraction 8, and the partial sequences of fractions 7 and 9.

The molecules are apparently not glycosylated.

Complete amino acid sequence of fraction 8 leads to the molecular mass of 5476, which corresponds well with the result of the MS: 5476.5.

25

The incomplete amino acid analysis of fraction 7 leads to, (supposing:

a) cysteines occur at the same positions as in fraction 8,

b) all cysteines are involved in S-S bridging,

30 c) Tyr-30 is the only substitution within residues 28 to 41 as compared to the sequence in fraction 8, —

d) No further substitutions occur after residue 41 as compared to the sequence in fraction 8,)

the calculation of a molecular mass of 5493, which corresponds

35 well with the result of the MS: 5494.4

The primary sequence is the following amino acid sequence of peak 8, which was found to be a single molecule.

(N-terminal): Asp-Asp-Asn-Cys-Gly-Gly-Lys-Val-Cys-Ser-Lys-Gly-
Gln-Leu-Cys-His-Asp-Gly-His-Cys-Glu-Cys-Thr-Pro-Ile-Arg-Cys-
Leu-Ile-Phe-Cys-Pro-Asn-Gly-Phe-Ala-Val-Asp-Glu-Asn-Gly-Cys-
5 Glu-Leu-Pro-Cys-Ser-Cys-Lys-His-Gln : (Carboxy-terminal)

Isoforms:

1. One other single molecule isoform was found in peak 7 (Fig. 7), its sequence from the N-terminus is:

5 Asp-Asp-Asp(Cys)Gly-Gly-Gln-Val(Cys)Ser-Lys-Gly-Gln-Leu(Cys)
His-Asp-Gly-His(Cys)Glu(Cys)Thr-Pro-Ile-Arg(Cys, Leu, Ile, Tyr,
Cys, Pro, Asn, ???, Phe, ???, Val, Asp, Glu, ???, ???)

2. Another isoform, which was found to be a mixture of two molecules was found in peak 9 (Fig. 7), starts from the N-terminus as follows:

Asp-Asp-Asp(Cys)Gly-Gly-Lys-Val(Cys)Ser-Lys-Gly-Gln-Leu-(Cys)
Gly Asn Gln
Val-Asp-Gly-His(Cys)Glu(Cys)Thr-Pro-Ile-Arg(Cys)Leu-Ile-Tyr-
15 Gln Lys
(Cys, Pro)Asn-Gly(Phe, Ala, Val, ???, Glu, Asn, Gly, Cys, ???, ???, ???)

Further research on the nature and the activities found in the fractions corresponding to the peaks of Fig. 7 revealed that the fractions corresponding to the three peaks 7, 8 and 20 9, had a specific (elastase) activity of 1.2 IU/mg, 1.7 IU/mg and 0.95 IU/mg, respectively.

International units (IU) are defined as that quantity which reduces the enzymatic hydrolysis of SAAP with 1 25 μ mol/min at pH 8.3 and 25°C.

The fraction corresponding to peak 5 of Fig. 7 which for obvious reasons seems of interest (it is the largest peak) was also tested for its elastase activity. Said specific activity turned out to be less than 0.06 IU/mg, which means that the 30 fraction corresponding to peak 5 contains no (hardly any) elastase-activity.

However, when further analyzing the fraction corresponding to peak 5, it revealed a strong activity in inhibiting trypsin. This trypsin inhibitory activity is at least twenty 35 times higher than that of the fractions corresponding to peaks 7, 8 and/or 9.

Trypsin inhibition is a very useful activity, therefore we analyzed the amino-acid sequences present in the fraction of peak 5. We found a 1:1 mixture of 2 peptides, which were analyzed by LD-MS in the same manner as the fractions corresponding to peaks 7, 8 and 9. The analysis revealed molecular weights for the two peptides of 5377.2 and 5435.5 Dalton.

Sequencing of the peptides revealed the following sequences:

10	1	5	10	15
	Asp- Asp - Asp -Cys-Gly-Gly-Gln-Val-Cys-Ser-Lys-Gly-Gln-Leu-Cys-			
	Gly Asn			
	16	20	25	30
	Val-Asp-Gly-Gln-Cys-Lys-Cys-Thr-Pro-Ile-Arg-Cys- Arg -Ile-Tyr-			
15	31	35	40	45
	Cys-Pro-Lys-Gly-Phe-Glu-Val-Asp?-Glu-Asn-Gly-Cys-Glu-Leu-Pro-			
	46	50		
	Cys-Thr-Cys-Lys?-Gln?			

20 The double residues indicated at positions two and three indicate the existence of the earlier mentioned mixture of two peptides.

These substances are named FAHSIN I 1/2.

25

Amino acid analysis of fraction 5 (Fig. 7) leads to, (supposing:

- a) cysteines occur at the same positions as in fraction 8,
- b) all cysteines are involved in S-S bridging,)

30 the calculation of a molecular mass of 5437 and 5387, which corresponds well with the result of the MS.

Amino Acid analysis of fraction 5 (Fig. 7) shows an unusual and unknown peak at between 10 and 12 minutes. It is speculated, that this may or may not be protein material, and may have certain biological effects not related to inhibition of Trypsin nor Plasmin.

35

An assay was developed for determination of the activity of this Trypsin inhibitor. In analogy with the elastase inhibition assay the preparation of the solution was as follows:

- 5 Trypsin, Type II-S soybean trypsin inhibitor (Sigma T 9128) and BAPNA (Sigma B 3279). This was further optimized to: trypsin solution: 120 µg/ml; BAPNA solution 10 mM; Trypsin inhibitor 100 µg/ml and incubation at 37 °C. All other details were equal with the elastase inhibition assay.
- 10 The (significant) replacements of residues in these two peptides when compared with the peptides of the fraction of peak 8 (and 7 and 9 as far as possible) are printed bold. These changes are position 28 Leu to Arg, position 33 Asn to Lys, position 36 Ala to Glu and possibly position 47 Ser to
- 15 Thr. When compared with the amino acid sequence of the fraction of peak 8, the length of these fraction 5 trypsin inhibitors may be reduced by one carboxy-terminal residue to a total of fifty residues.
- 20 Methods for de novo synthesis.

In order to obtain sufficient quantities of the compositions of matter according to the invention, one can use known gene technological methods (see: Sambrook, T. et al:

25 Molecular cloning: A laboratory Manual, Cold Spring Harbor Press, N.Y., USA., 1989). Four well-known methods, forming part of the known state of art, can be used for such synthesizing of the composition of matter.

First: Chemical addition technique in which the various

30 amino acids are added: peptide synthesis (see Merrisfield).

Second: After the synthesizing of an oligonucleotide, which bases correspond to the amino acid sequence as defined in this invention, such oligonucleotide is consequently built into a plasmid vector system, which then is brought into a useful

35 bacterial or fungal carrier, which is then grown. Finally the synthesized molecule is retrieved from the cultures.

Third: The method for production and hybridisation of cDNA-libraries is in the art (see: Sambrook, T, et al, Molecular cloning: A laboratory Manual, Cold Spring Harbor Press, N.Y.USA., chapters 7, 8 and 11, 16 and 17). After elaboration
5 of such a DNA library, identification of the genome which is coding for the protein sequence is searched. Thereafter, according to wellknown methods, the genome can be expressed in eukaryotic cells, yeasts, bacteria, and the protein can be acquired in larger quantities.

10 Fourth: The cells producing the protein can be cultivated in a monoculture and the protein is derived therefrom.
HIV-inhibition.

Fahsin was found to be active against HIV-1 and HIV-2
15 isolates in peripheral blood mononuclear cells (PBMC) and in primary macrophages.

Experimental:

20 Phytohaemagglutinin (PHA) stimulated PBMC from healthy donors were inoculated with two different HIV isolates (HIVams 37 and HIVams 55). After a two-hour exposure to the HIV isolates, the inoculum was removed and serial concentrations Fahsin were added to the cultures. Medium was changed twice a
25 week, fresh PMA-stimulated PBMC were added every week. Buffy-coats are routinely screened for viral contaminants and used only when negative for these contaminants. Virus production in the cultures was monitored with a p24-capture ELISA, detecting p24 core protein of HIV. Cultures were also
30 monitored for the occurrence of cytopathic effects (syncytium formation).

High titre inocula of two primary syncytium inducing HIV isolates were prepared. Titers of stock were determined in a TCID50 assay.

35 During the first two days after isolation of the cells, primary peripheral blood leukocytes were cultured in Iscove's Modified Dulbecco's Medium (IMDM), supplemented with 10%

foetal calf serum (FCS), polybrene (5(g/ml),
phytohaemagglutinin (5(g/ml), penicillin (100 IU/ml) and
streptomycin (100 IU/ml). The T-cell blasts were then further
cultured in IMDM supplemented with 10% FCS, polybrene
5 (5(g/ml), recombinant IL-2 (10 U/ml), penicillin (100 IU/ml)
and streptomycin (100 IU/ml).

A total of 107 PHA-stimulated PBMC were inoculated with
104 TCID50/ml of the primary HIV isolates in a volume of 1 ml
for 2 hours at 37°C. After 2 hours, cells were washed in a
10 total volume of 30 ml. After centrifugation, supernatant was
discarded to remove non-absorbed virus. Cells were
subsequently resuspended to a final concentration of 106/ml.

From each cell suspension 100 µl aliquots containing 105
cells were transferred to wells of a 96 well tissue culture
15 plate. Dilutions of Fahsin were made in culture medium in such
a way that after addition of 50 µl to each well the final
concentrations in the wells was 0.125 µM, 0.25 µM, 0.5 µM and
1.0 µM. Cells that received only medium served as untreated
control cultures. Each concentration was analyzed in four
20 fold. Cells were cultured in a humidified atmosphere at 37°C,
5% CO₂. Addition of 105 fresh PHA stimulated PBMC and medium
was performed on day 7. In parallel, a new dose of the same
concentrations of Fahsin was added.

Controls: On days 4 and 7, cultures were analyzed for any
25 HIV-induced cytopathic effect as reflected by the presence of
syncytia. At days 7, and 14, 30 µl of the culture supernatant
was harvested to analyze the presence of p24 antigen in a p-
24-antigen-capture-ELISA. For this, twice a week, 30 µl
aliquots were harvested from the cultures and inactivated by
30 the addition of 30 µl 0.2% Triton-X-100. 15 µl of this mixture
was added to wells of a 96 well ELISA plate coated for 2 hours
at 37°C with an anti-p24 antibody shown to recognize all HIV
isolates. Antigen was allowed to bind during 2 hours at 37°C.
Bound p24 was detected with horseradish peroxidase-labelled
35 rabbit-anti-p24 immunoglobulin (90' at 37°C). Then, substrate
(TMB) was added and after 20', the reaction was stopped by the
addition of H₂SO₄.

Results are presented in Figs. 8a through 8d.

The two Fahsin substances corresponding to the tested substances are:

Substance 1: Combination of Fahsin E 1-4 and Fahsin T 1-2.

5 Substance 2: Total leech body extract as presented under the heading "description of isolation".

Results showed that both substances have inhibitory effects on the replication of two primary isolates HIVams 37 and HIVams 55.

10 The results are arrived at in the following manner:

CALCULATION OF RESULTS

For the calculation of the percentage inhibition the following formula was used:

15

Percentage inhibition =

$$20 \quad \frac{\text{p24 production in untreated cultures} - \text{p24 production in cultures exposed to substance}}{\text{p24 production in untreated cultures}} \times 100\%$$

CRITERIA

25 Cultures were considered positive if:

Syncytia formation was observed on at least one occasion (cultures were examined twice a week) in combination with an elevated p24 antigen content in the supernatant on at least
30 one occasion.

Syncytium score:

- no syncytia observed in any of the four replicate cultures
- ± syncytia observed in one out of four replicate cultures
- 35 + syncytia observed in two out of four replicate cultures
- ++ syncytia observed in three out of four replicate cultures
- +++ syncytia observed in four out of four replicate cultures

RESULTS

5 HIV-1 induced cytopathic effects (CPE), production of p24 antigen, and calculated percentages inhibition are demonstrated in Tables 1a and 1b for substance 1, in Tables 1c and 1d for substance 2.

10 Data for p24 production represent the mean of four replicate cultures.

Cultures inoculated with 10^2 TCID₅₀ remained negative for virus production.

Comparison with known molecules.

Comparison of the molecule (1) with known molecules from databanks gave the following results:

No homology, nor identical peptide structure was found
 5 between the sequences of Fahsin, Eglin (Seemueller), and Gelin (PCT/NL90/46).

1. With Antistasin- Hydra magnipapillata (S29195): 15
 derivates identity (29.4%) in 29 aa overlap.
2. With Transforming growth factor beta-1 binding protein
 10 (A35626): 15 derivates identity (29.4%) in 31 aa overlap.
3. With Fibrillin, human (L13923 and X63556): 17 derivates
 identity (33.3%) in 52 aa overlap.
4. With LDL receptor-related protein precursor, human
 (S02392): 10 derivates identity (19.6%) in 29 aa overlap.
- 15 5. Alpha-2-macroglobulin receptor, mouse (S25111): 10
 derivates identity (19.6%) in 29 aa overlap.
6. With VLDL receptor, human (D16493 and D 16494): 7
 derivates identity (13.7%) in 11 aa overlap.
7. With Alpha-2-macroglobulin receptor, human (S30027): 10
 20 derivates identity (19.6%) in 29 aa overlap.
8. With thrombospondin precursor, human (A26155): 12
 derivates identity (23.5%) in 48 aa overlap.
9. With Anticoagulant Protein Ghilanten, H. Ghilianii
 (A34816) 16 derivates identity (31.4%) in 46 aa overlap.
- 25 10.
11. With Complement Pro-C3 precursor, human (A37156) 10
 derivates identity (19.6%) in 25 aa overlap.
12. With Hepatitis C-virus mRNA (M96362) 8 derivates identity
 (15.7%) in 17 aa overlap.
- 30 13. With Antistasin, from Haementeria officinalis (A34398 &
 S13904): 17 derivates identity (33.3%) in 47 aa overlap.

Stability of the molecule Fahsin E2.

- 35 Stability of the molecule was tested
- a) by boiling it in water at 100°C for 30 minutes.

b) by immersion in 50% acetic acid at room temperature during one night, and

c) by immersion in 0.1 M HCl in the same conditions.

The samples were consequently assayed with the elastase inhibition assay, as were blancs without inhibitor.

Results have shown no significant decrease of inhibiting activity in the samples. Therefore, it was concluded, that this molecule is extremely resistant to degradation by high temperatures and strong acid.

Possible active sites.

A database search was performed to estimate the probability for the active site. Prediction of structure resulted in:

No Helical conformation,
15.6% in extended conformation, and
84.3% in coil conformation

A predicted beta-turn was likely to be the active site, as is usual with most serpins (exposed binding loop). Aa 28-32 have a high probability for such an extended conformation, with residue 28 being the most exposed. It is known, that elastase inhibitors often have a Pro at P4', whereas trypsin inhibitors have an Arg at P1. We have concluded provisionally that the active site resides in Leu/Arg 28.

Manufacturing of a synthetic peptide inclusive of the proposed active site gave the following evidence.

Synthetic linear peptide: N-Acetyl-TPIRAbuLIFAbuPNGFAVD-amide(I), mimicking the residues 23 to 38 included, as elastase inhibitor and

Synthetic linear peptide: N-Acetyl-TPIRAbuRIYAbuPKGFEVD-amide(II) mimicking the residues 23 to 38 included, as trypsin inhibitor, were produced from the C-terminus to the N-terminus on a 10(mol scale using solid-phase Fmoc chemistry. The crude peptides are partly purified by several ether-precipitations. A 15-mer, 10 mg of partly purified product is obtained. From

this 10 mg a part of 7 mg consists of peptide material, of which at least 50% of the desired full length product, and a part of 3 mg of salts and remaining solvent (mainly water).

The quality of the final product was checked by sequence
5 analysis, aminoacid analysis, LD-MS and RP-HPLC.

Synthetic peptide I (elastase inhibitor) shows a dose-dependent inhibition of elastase activity. Specific effectivity is about a factor 5,000 less than the native molecule, which is indicating both the active site and high
10 efficiency of the synthetic peptide.

Synthetic peptide II (trypsin inhibitor) shows a dose-dependent inhibition of trypsin activity. Specific effectivity is about a factor 1,500 less than the native molecule, which is indicating both the active site and an even higher
15 efficiency of the synthetic peptide.

Range of activities.

Fahsin E 1-4 was found to be strongly active as an
20 inhibitor of human neutrophil elastase, pancreas elastase, chymotrypsin, but not of pepsin. Fahsin T 1-2 was found to be strongly active as an inhibitor of trypsin and plasmin. Fahsin was found to have strong antibiotic activity against Aeromonas species. Elastase inhibitory activity of Fahsin was measured
25 by inhibition of the release of the p-nitroanilide group from the synthetic substrate N-succinyl-(Ala) 3-p-nitroanilide (SAAAP) (Calbiochem), catalyzed by pancreatic elastase and human neutrophil elastase respectively. Chymotrypsin inhibition activity of Fahsin was measured by the use of the
30- synthetic substrate S-2586 (Kabi Vitrum). Trypsin activity of Fahsin was measured by the use of the synthetic substrate S-2238 (Kabi Vitrum). pepsin activity of Fahsin was measured by the use of a haemoglobin substrate. Trypsin inhibition activity was determined using the BAPNA assay. Plasmin
35 inhibition activity of Fahsin was related with the Trypsin inhibition activity and measured by the use of the chromogenic substrate D-Val-Leusyl-Lys-pNA (Othodiagnostics).

Fahsin was also found to be active against HIV-1 and -2 replication in peripheral blood mononuclear cells (PBMC) and in primary macrophages. The inhibition was experimentally achieved by inoculation of PBMC from healthy donors with HIV isolates HIVAms 37 and HIVAms 55.

Inhibition was detected by the occurrence of HIV-induced cytopathic effects (syncytium formation) in cultures with dilution series of the various Fahsin substances, as well as the p24 antigen capture ELISA.

Fahsin was also found to be able to dissolve fibrin. This was measured by the use of Cow's thrombin (Sigma T 6634) which was incubated with Cow's fibrinogen (Miles 82-0222-4), leaving stable fibrin clots. After incubation of this clot with the protein, which subsequently led to the measurement of free protein substance. This is in agreement with our macroscopic observations of clot-lysing potential of the live leech.

Fahsin was also found to be an effective thrombin inhibitor. This activity was determined by measuring the inhibition of the clotting activity of thrombin upon fibrinogen as was earlier described (Markwardt, 1970).

All substances Fahsin from *L nilotica* have low potential for antigenic effect, as was observed from the remarkable way of living of this leech. Therefore, therapeutic use of (natural) molecules from *L nilotica* as described herewith, -and (natural) molecules from *L nilotica* still being determined-, as well as natural and synthetic mimicks of these natural molecules, will have a low immunogenicity.

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CLAIMS.

1. A substance having protease inhibiting activity obtainable from *Limnatis Nilotica* or a fragment or derivative of such a substance having similar activity.
2. A substance according to claim 1 which is proteinaceous or
5 polypeptide-like.
3. A proteinaceous or polypeptide-like substance according to claim 2, which comprises at least part of one of the following amino acid sequences
DDNCGGKVCCKGQLCHDGHCECTPIRCLIFCPNGFAVDENGCELPCCKHQ
10 DDDCGGQVCKGQLCVDGQCKCTPIRCRIYCPKGFVDENGCELPCTCLQ
DGNCGGQVCKGQLCVDGQCKCTPIRCRIYCPKGFVDENGCELPCTCLQ
or such a sequence which contains one or more conventional amino acid substitutions.
4. A substance according to anyone of the foregoing claims
15 which in particular inhibits elastase, chymotrypsin, trypsin or thrombin, plasmin.
5. A substance according to anyone of claims 1-3, which dissolves fibrin/fibrinogen.
6. A substance according to anyone of claims 1-3, which
20 inhibits the replication of human immunodeficiency virus in human cells.
7. A substance according to anyone of claims 1-3, having antibiotic activity.
8. A substance according to any one of the foregoing claims
25 for use as a therapeutic.
9. A pharmaceutical composition comprising a substance according to anyone of claims 1-7 together with suitable excipients.
10. Use of a substance according to anyone of claims 1-7 in
30 the preparation of a medicament for the treatment of Diabetes Mellitus, blood clotting disorders, disorders of neutrophil function, rheumatoid arthritis, Human Immunodeficiency Virus infections, or other immunological or inflammatory disorders.

11. A nucleic acid molecule encoding at least part of a proteinaceous or polypeptide-like substance according to claim 3.

12. A nucleic acid molecule according to claim 11, comprising
5 at least part of the following sequence

CTR CTR TTR ACR CCN CCN TTY CAN ACR AGN TTY CCN GTY AAY ACR GTR
CTR CCN GTR ACR CTY ACR TGN GGN TAD GCN ACR AAY TAD AAR ACR CGN
TTR CCN AAR CGN CAN CTR CTY TTR CCN ACR CTY AAY GGN ACR AGN ACR
TTY GTR GTY

10 whereby R=A/G, N=A/C/G/T, Y=C/T, D=A/G/T,
or a sequence having 70% homolgy therewith.

13. An expression vector comprising a nucleic acid molecule according to claim 11 or 12 together with suitable control elements for expression.

15 14. An expression system for expressing at least part of a substance according to claim 3, comprising an expression vector according to claim 13 and an expression mechanism.

15. An expression system according to claim 14, wherein the expression system is a recombinant host cell.

20 16. An antibody which specifically recognizes a substance according to anyone of claims 1-7.

17. An anti-idiotypic antibody mimicking at least an epitope of a substance according to any one of claims 1-7.

18. An anti-idiotypic antibody mimicking at least an activity
25 of a substance according to any one of claims 1-7.

— A233
— A280

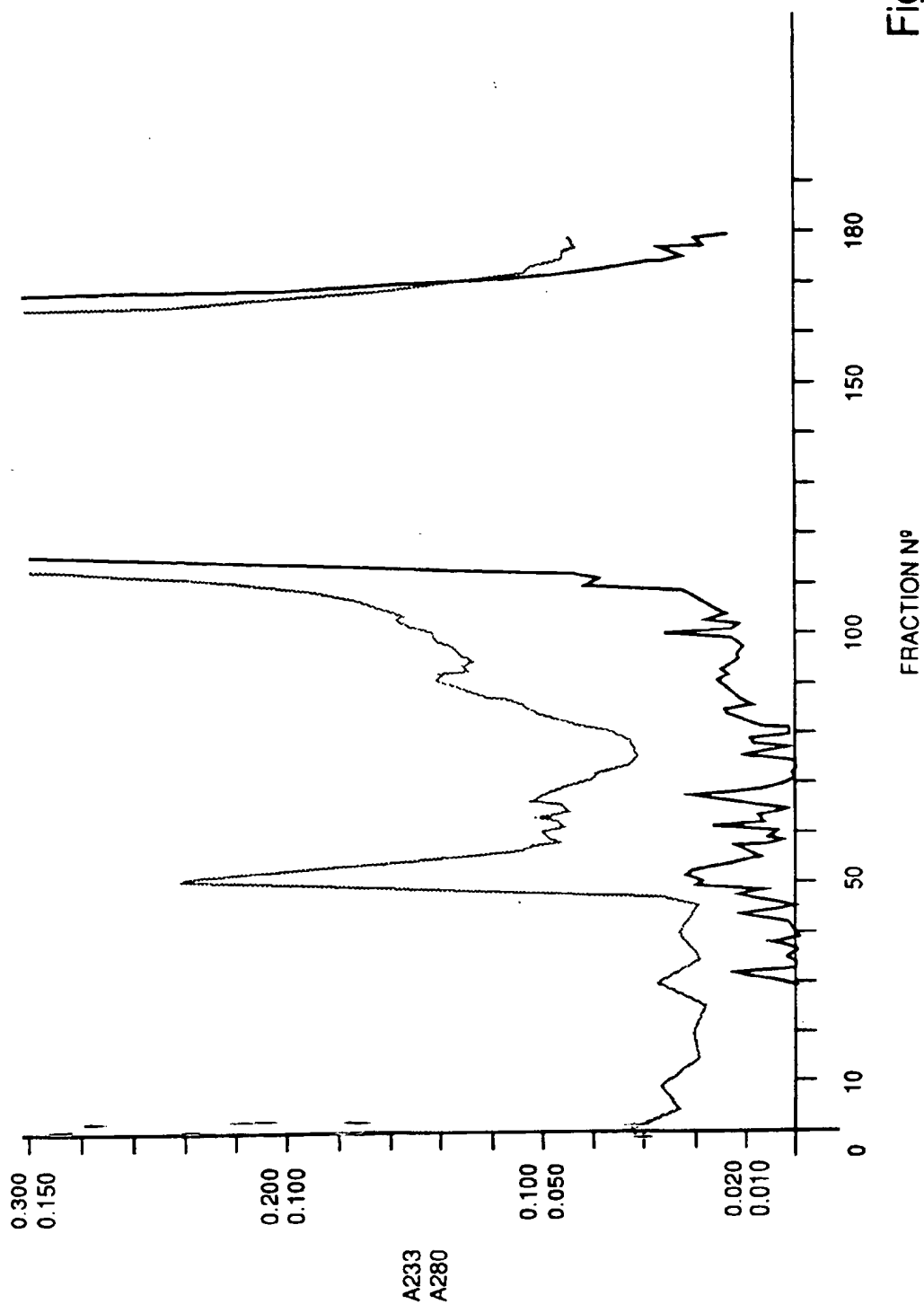


Fig. 1

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Fig. 2a

No.	A280	A233	No.	A280	A233
30	0,001	.056	62	.007	.096
31	0,0 10	.056	63	.007	.102
32	0.012	.051	64	.002	.090
33	-0,003	.047	65	.003	.093
34	-0,002	.047	66	.018	.105
35	0.002	.049	67	.020	.101
36	-0,002	.044	68	.009	.099
37	-0,001	.045	69	.004	.092
38	0.005	.049	70	.001	.082
39	.002	.040	71	.001	.081
40	-.001	.047	72	.000	.073
41	-.001	.043	73	.000	.068
42	.002	.042	74	.000	.063
43	.013	.042	75	.010	.063
44	.006	.043	76	.002	.064
45	.000	.040	77	.008	.065
46	.003	.042	78	.009	.066
47	.013	.054	79	.002	.072
48	.007	.114	80	.002	.075
49	.020	.208	81	.007	.087
50	.019	.242	82	.010	.094
51	.022	.240	83	.014	.102
52	.021	.217	84	.014	.106
53	.018	.182	85	.009	.109
54	.013	.152	86	.012	.116
55	.007	.126	87	.013	.125
56	.009	.109	88	.014	.130
57	.013	.104	89	.015	.136
58	.003	.096	90	.016	.142
59	.006	.099	91	.014	.140
60	.005	.100	92	.015	.135
61	.017	.093	93	.013	.131

A 280/233 absorption of Sephadex fractions 30-93

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Fig. 2b

No.	A280	A233	No.	A280	A233
94	.012	.129	155	1,651	>1
95	.012	.132	156	1,464	1,595
96	.011	.135	157	1,280	1,250
97	.011	.136	158	1,120	1,032
98	.013	.140	159	0,953	.863
99	.026	.143	160	0,815	.736
100	.013	.146	161	.662	.595
101	.012	.153	162	.543	.500
102	.018	.157	163	.437	.415
103	.014	.150	164	.351	.352
104	.015	.162	165	.272	.290
105	.017	.167	166	.217	.249
106	.018	.174	167	.163	.206
107	.020	.182	168	.130	.183
108	.023	.197	169	.099	.160
109	.041	.217	170	.079	.146
110	.039	.240	171	.061	.123
111	.045	.263	172	.048	.110
112	.060	.304	173	.038	.100
113	.089	.370	174	.033	.102
114	.045	.484	175	.026	.095
115	.226	.685	176	.020	.091
116	.400	1,111	177	.027	.090
117	.660	>1	178	.018	.086
118	.994	>1	179	.019	.089
119	1.326	>1	180	.014	.082
120	1.607	>1	1		.066
121	1.902	>1	5		.048
122	>1	>1	10		.054
123	>1	>1	15		.040
125	>1	>1	20		.042
130	>1	>1	25		.048

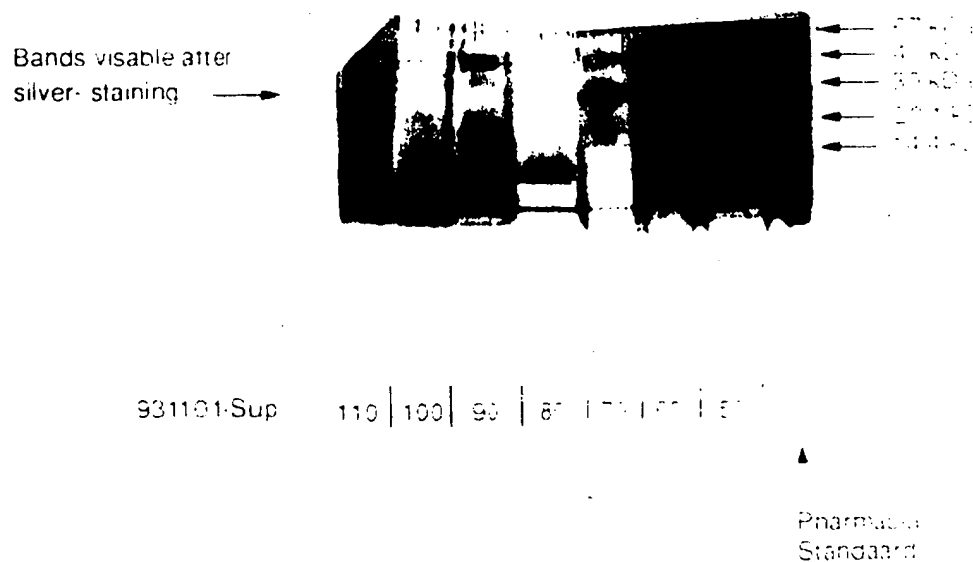
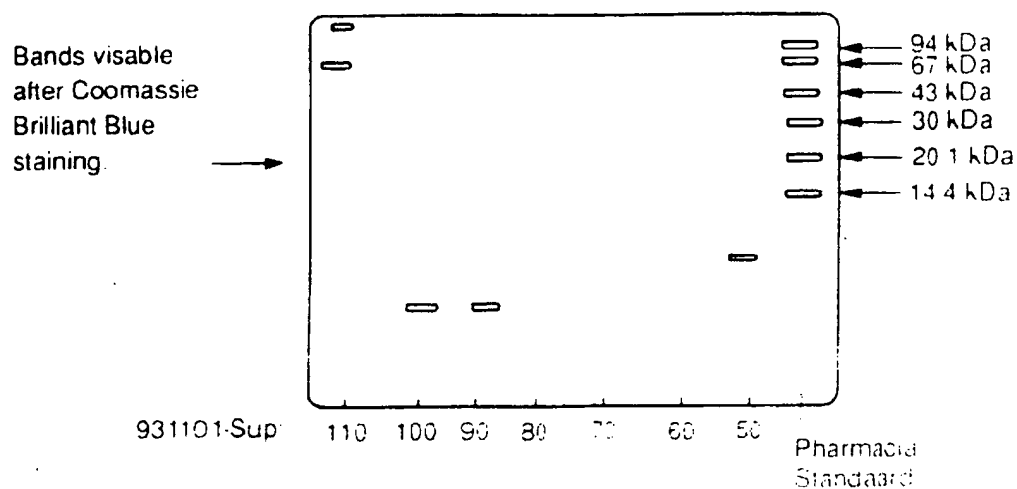
A 280/233 absorption of Sephadex fractions 94-180; and 1-25

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fig. 3

Fractio	A105	Fractio	A105	
B+	0.450	156	0.172	
B-	0.005	160	0.198	
48	0.164	164	0.156	
52	0.166	168	0.163	
56	0.165	172	0.157	
60	0.161	176	0.171	
64	0.171	180	0.177	
68	0.167			B+ = blank
72	0.171			0.1 M acetic acid,
76	0.167			with SAAAP
80	0.168			B- = blank
84	0.172			0.1 M acetic acid,
88	0.172			without SAAAP
92	0.132			
96	0.014			
100	0.008			
104	0.007			
108	0.006			
112	0.015			
116	0.156			
120	0.183			
124	0.179			
128	0.189			
132	1.194			
136	0.198			
140	0.190			
144	0.190			
148	0.177			
150	0.223			

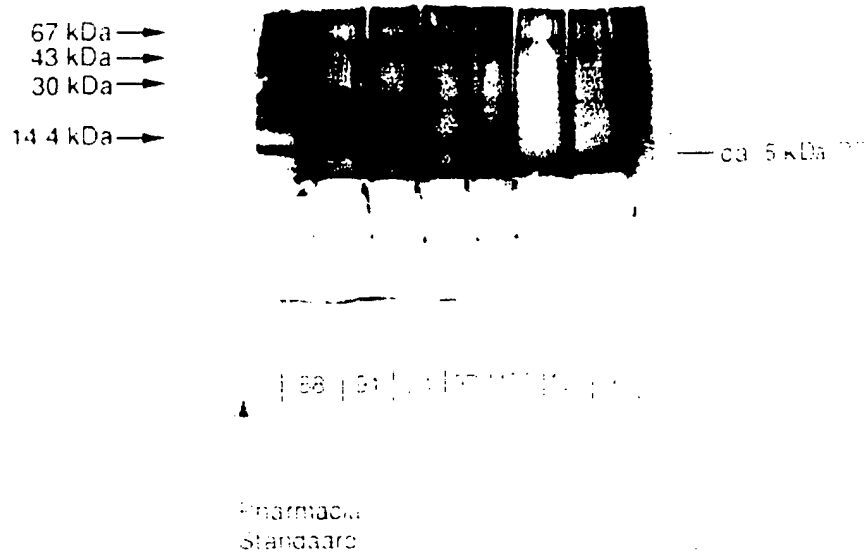
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SPS-PAGE of Sepadex G-75 fractions of
931101-Sup
 (GEL-1)

Fig. 4

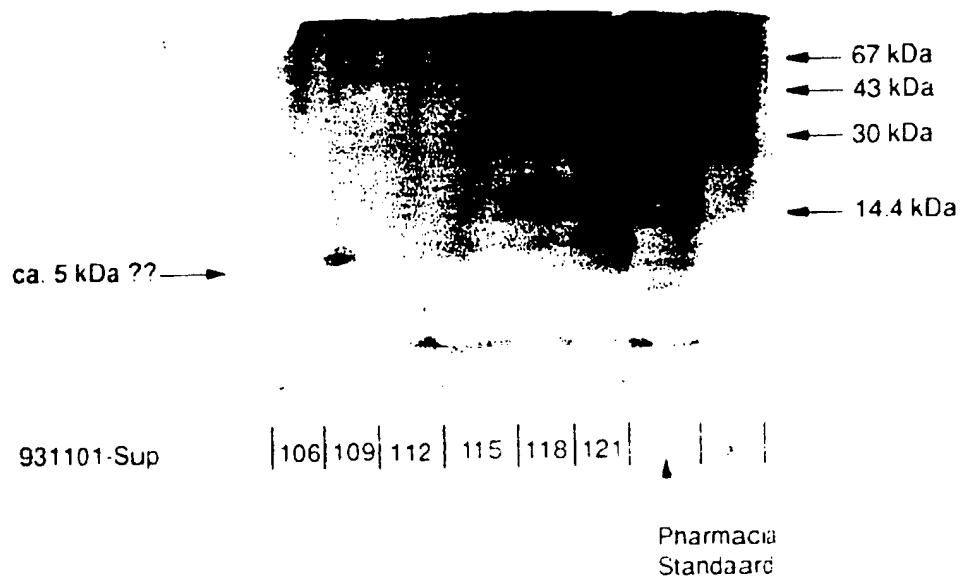
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SPS-PAGE of Sepadex G-75 fractions of
931101-Sup
(GEL-2)

Fig. 5

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SPS-PAGE of Sepadex G-75 fractions of
931101-Sup
(GEL-3)

Fig. 6

LC A 214,10 350,20 of 2903F40A.D

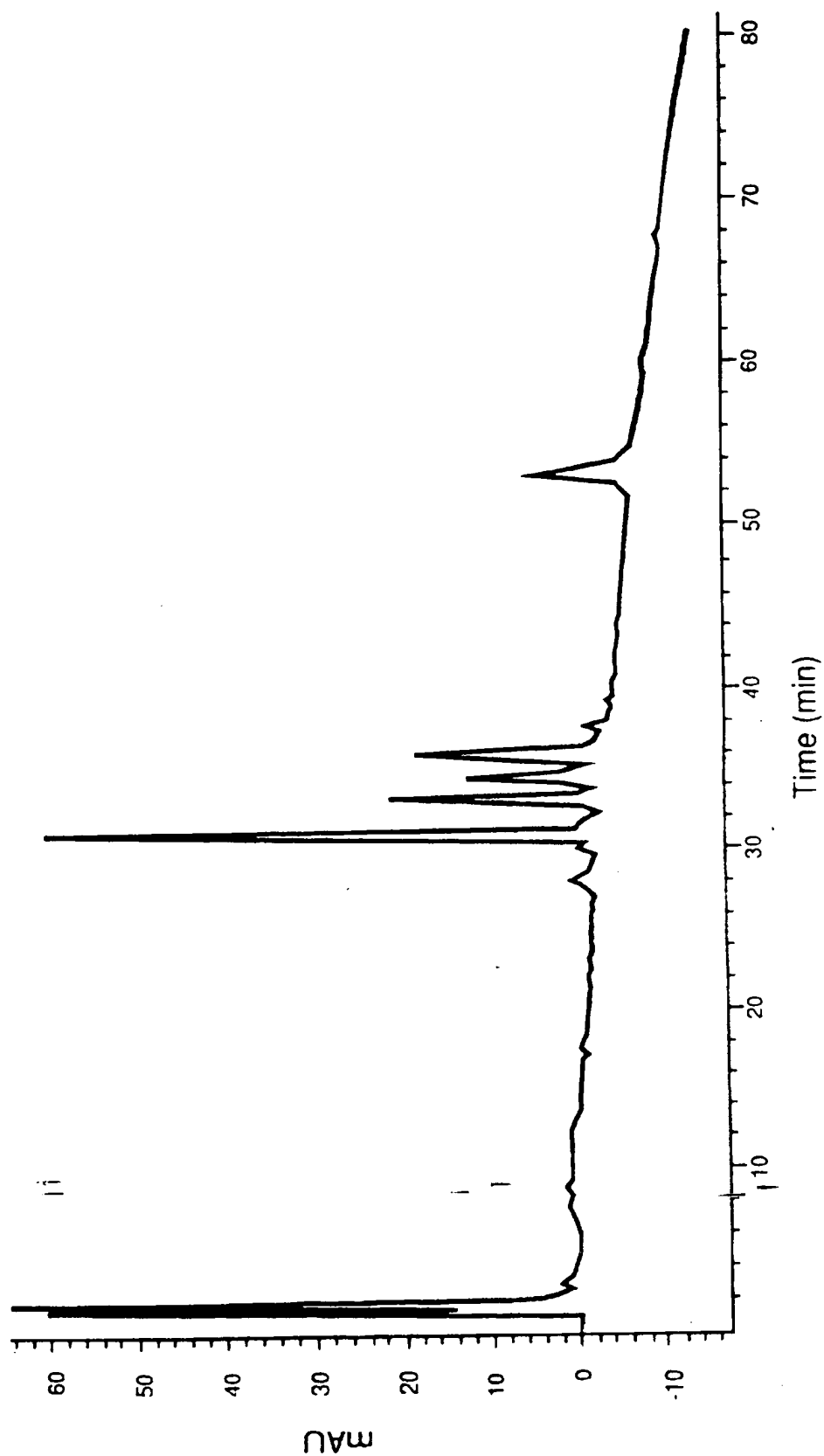


Fig. 7

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Fig. 8a
Substance 1

Primary HIV-1 isolate HIV _{mn} 37						
Inoculum (TCID ₅₀ /ml):	10 ⁴					
Concentration of substance (μM)	CPE		Day 7		Day 14	
	Day 4	Day 7	p24 (μg/ml)	Percentage Inhibition	p24 (μg/ml)	Percentage Inhibition
0	++	+++	1.00		0.56	
0.125	+++	+++	0.70	30	0.48	14
0.25	+++	+++	0.72	28	0.45	20
0.5	++	+++	0.65	35	0.46	18
1.0	±	+++	0.60	40	0.38	32

Fig. 8b
Substance 1

Primary HIV-1 isolate HIV _{mn} 55						
Inoculum (TCID ₅₀ /ml):	10 ⁴					
Concentration of substance (μM)	CPE		Day 7		Day 14	
	Day 4	Day 7	p24 (μg/ml)	Percentage Inhibition	p24 (μg/ml)	Percentage Inhibition
0	+++	+++	1.43		0.63	
0.125	+++	+++	1.25	13	0.64	-2
0.25	++	+++	1.05	27	0.42	33
0.5	+++	+++	1.16	19	0.51	19
1.0	+++	+++	1.00	30	0.41	35

10/10

Fig. 8c

Substance 2

Primary HIV-1 isolate HIV _{nu} 37						
Inoculum (TCID ₅₀ /ml):	10 ⁴					
Concentration of substance (μM)	CPE		Day 7		Day 14	
	Day 4	Day 7	p24 (μg/ml)	Percentage Inhibition	p24 (μg/ml)	Percentage Inhibition
0	++	+++	0.66		0.41	
0.125	-	+++	0.67	0	0.39	5
0.25	-	+++	0.61	8	0.31	24
0.5	++	+++	0.55	17	0.26	37
1.0	++	+++	0.55	17	0.24	41

Fig. 8d

Substance 2

Primary HIV-1 isolate HIV _{nu} 55						
Inoculum (TCID ₅₀ /ml):	10 ⁴					
Concentration of substance (μM)	CPE		Day 7		Day 14	
	Day 4	Day 7	p24 (μg/ml)	Percentage Inhibition	p24 (μg/ml)	Percentage Inhibition
0	--	+++	1.25		0.45	
0.125	---	+++	1.25	0	0.45	0
0.25	---	+++	1.19	5	0.36	20
0.5	---	+++	1.23	2	0.42	7
1.0	--	+++	1.14	9	0.37	18

INTERNATIONAL SEARCH REPORT

International Application No

PC/EP 95/04223

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/15 C07K14/815 A61K38/58 C07K16/38

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 348 208 (YISSUM RESEARCH DEVELOPMENT COMPANY OF THE UNIVERSITY OF JERUSALEM) 27 December 1989 cited in the application see page 9, last paragraph - page 10; claim 2; table 3 ---	1,2, 8-10, 16-18
Y	EUR. J. BIOCHEM., vol. 219, 1994 pages 937-943, SÖLLNER C. ET AL. 'Isolation and characterization of hirustasin, an antistatin-type serine-proteinase inhibitor from the medical leech Hirudo medicinalis' see figures 5,6 --- -/--	1,2,4, 8-10, 16-18

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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- *A* document defining the general state of the art which is not considered to be of particular relevance
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- *P* document published prior to the international filing date but later than the priority date claimed

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- *Z* document member of the same patent family

Date of the actual completion of the international search

20 March 1996

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26.03.96

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 166, no. 3, 1990 DULUTH, MINNESOTA US, pages 1384-1389, BLANKENSHIP D. T. ET AL. 'Amino acid sequence of ghilanten: anticoagulant-antimetastatic principle of the south american leech, Haementeria ghilianii' cited in the application see figure 2 ---	1,2,4, 8-10, 16-18
P,X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 23, June 1995 MD US, pages 13879-13884, JUNG H. I. ET AL. 'Isolation and characterization of guamerin, a new human leukocyte elastase inhibitor from Hirudo nipponia' see the whole document -----	1-4, 11-15

information on patent family members

Inter-Office Ref No

PL 115-4223

Form PCT/ISA:210 (patent family annex) (July 1992)